Published in final edited form as:

J Immunol. 2017 February 01; 198(3): 1263–1273. doi:10.4049/jimmunol.1600093.

Staphylococcus aureus Protein A Disrupts Immunity Mediated by Long-Lived Plasma Cells[1]

Amanda B. Keener[*], **Lance T. Thurlow**[†], **SunAh Kang**[*], **Nicholas A. Spidale**[‡], **Steven H. Clarke**[*], **Kenji M. Cunnion**[§], **Roland Tisch**[*], **Anthony R. Richardson**[†], and **Barbara J. Vilen**[*]

[*]Department of Microbiology and Immunology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[†]Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA, USA

[‡]Department of Pathology, Massachusetts Medical School, Worcester, MA

[§]Department of Microbiology and Molecular Cell Biology and the Department of Physiological Sciences, Eastern Virginia Medical School, Norfolk, VA, USA

Abstract

Infection with *Staphylococcus aureus* does not induce long-lived protective immunity for reasons that are not completely understood. Human and murine vaccine studies support a role for antibodies in protecting against recurring infections, but *S. aureus* modulates the B cell response through expression of Staphylococcal Protein A (SpA), a surface protein that drives polyclonal B cell expansion and induces cell death in the absence of co-stimulation. In this murine study, we show that SpA altered the fate of plasmablasts and plasma cells (PCs) by enhancing the short-lived extrafollicular response and reducing the pool of bone marrow (BM)-resident long-lived PCs (LLPCs). The absence of LLPCs was associated with a rapid decline in antigen-specific, class-switched antibody. In contrast, when previously inoculated mice were challenged with isogenic Δ*spa S. aureus*, cells proliferated in the BM survival niches and sustained long-term antibody titers. The effects of SpA on PC fate were limited to the secondary response, as antibody levels and the formation of B cell memory occurred normally during the primary response in mice inoculated with either WT or Δ*spa S. aureus*. Thus, failure to establish long-term protective antibody titers against *S. aureus* was not a consequence of diminished formation of B cell memory; instead, SpA reduced the proliferative capacity of PCs that entered the BM, diminishing the number of cells in the long-lived pool.

[1]This work was supported by the National Institute of Health Grant R01 AI070984 and NIH R21 AI105613 to BJV. ABK was supported by 5 T32 AI 7273-29 and through the UNC Program in Translational Medicine by a Howard Hughes Medical Institute (HHMI) grant supporting the Med into Grad Initiative. The UNC Flow Cytometry Core Facility, the Microscopy Services Laboratory, and the Animal Hisotpathology Core are supported by an NCI Center Core Support Grant (P30CA016086) to the UNC Lineberger Comprehensive Cancer Center.

Corresponding author: Barbara J. Vilen, Department of Microbiology & Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, barb_vilen@med.unc.edu, Phone: (919) 966-5436, Fax: (919) 962-8103.

Introduction

Staphylococcus aureus is a major cause of hospital and community acquired infections and has become more difficult to treat as antibiotic-resistant strains spread (1). *S. aureus* infections commonly recur without inducing long-term immunity (2) and despite the success of some vaccine approaches at inducing short-term protection in mouse models, attempts to design a human vaccine have failed (3–5). Studies in humans and mice have correlated pre-existing serum antibody (Ab) and passive immunization with improved clinical and experimental outcomes (6–10). Mutations that reduce Ab production also increase the risk of acquiring *S. aureus* infections (5). Further, a recent study reported that maintenance of antigen-specific Ab during convalescence correlated with reduced recurrence of infection (11). Despite this evidence that B cells can play a role in protection against *S. aureus*, little is known about the long-term humoral response to *S. aureus*.

Staphylococcus Protein A (SpA) is a virulence factor released from *S. aureus* during normal cell division allowing soluble and processed antigens to activate the immune system (12, 13). SpA activates B cells upon binding the Fab portion of B cell receptors (BCRs) of the V_H3 clan (up to 50% of naïve B cells in humans and 5–10% in mice) inducing robust non-specific B cell response (14–16). SpA sequesters IgG by binding the Fc portion and interferes with immune complex (IC)-mediated antigen presentation and phagocytosis (17, 18). This suggests that the expression of SpA on *S. aureus* alters the B cell response to infection.

Memory B cells and long-lived plasma cells (LLPCs) confer long-term humoral immunity. Serum Ab levels are sustained by LLPCs derived mainly from memory B cells activated during secondary immune responses (19–21). Memory B cells have been identified after infection with viral, parasitic, and bacterial pathogens (22–25), but whether these cells are formed after *S. aureus* infection has not been directly addressed. Memory B cells reside in an inactive state in secondary lymphoid organs, and upon re-exposure to antigen, rapidly divide into daughter cells or differentiate into antibody secreting cells (ASCs), which include surface Ig positive dividing plasmablasts (PBs) and the more mature, non-dividing surface Ig^- plasma cells (PCs) (26). Though the majority of PBs and PCs are short-lived, some migrate to survival niches in the bone marrow (BM), proliferate, and mature into LLPCs (19, 21, 27). Niche factors, including APRIL, support PB proliferation, and PC differentiation and survival (28–30). LLPCs and their constitutively secreted Ab are recognized as major contributors to protection against bacterial infection (31–33). In mice, LLPCs can survive the lifetime of an organism while in humans, they can survive for months to decades, and are the main source of convalescent serum IgG (20, 34, 35). It remains unclear whether LLPCs are formed after *S. aureus* infection, or whether SpA influences long-term B cell memory or LLPCs.

In this study, we show that SpA disrupts the proliferation of PBs in the BM and subsequent accumulation of LLPCs, while promoting expansion of short-lived PBs in the spleen. Mice previously inoculated and then challenged with a SpA-deficient *S. aureus* mutant (Δ*spa*), but not with WT *S. aureus,* formed LLPCs and maintained serum Ab for at least 12 weeks after challenge. The lack of long-term humoral immunity to WT *S. aureus* was not due to defects

in memory B cell formation or activation because primary inoculation with either WT or Δ*spa* induced the formation of germinal centers (GC) and functional memory B cells. Rather, SpA altered the differentiation of ASCs during the secondary response by expanding IgM^+ extrafollicular PBs, and reducing the proliferative capacity and the expression of the survival receptor, CD138 on PBs that entered the BM, thereby diminishing their ability to enter the LLPC pool.

Materials and Methods

Mice

C57BL/6 mice were bred and maintained in a specific pathogen-free facility at UNC Chapel Hill according to the Institutional Animal Care and Use Committee standards. At 7–8 weeks of age, mice were inoculated subcutaneously (sc) with 1×10^8 colony forming units (CFUs) of *S. aureus* RN4220 (Δ*spa* or WT), Newman, or USA300 in 20 μl of 1x PBS. For challenge experiments, 5 weeks after primary inoculation, 1×10^6 CFU were delivered intravenously (iv) via tail vein injection in 100 μl of 1x PBS. For NP-KLH experiments, 100 μg of NP_{20}KLH conjugated to alum (Imject) was delivered by intraperitoneal injection (ip). Immunized mice were boosted (iv) with 100 ug of soluble NP_{20}KLH.

Bacteria

S. aureus RN4220 (Δ*spa* and WT), Newman and USA300 were grown in brain heart infusion (BHI) broth at 37°C overnight and washed and diluted in 1x PBS for inoculations. CFUs were confirmed by plating serial dilutions on BHI plates. Isogenic *S. aureus* Δ*spa* mutants (Newman and RN4220) were constructed by amplifying ≈ 1 kb homology fragments flanking *spa* with primers Spa5′.1A (CGACTCTAGAGGAAAAACACACTTC) and Spa5′.1B (TAAGGATCGGGGACAATACCTACACC) for amplification of the 5′ homology fragment and primers Spa3′1A (TACCGAGCTCGAATATCATTTTATCC) and Spa3′1B (CAGTGCAGCGGAATTGCTTGGAGTCC) for amplification of the 3′ homology fragment. The resulting fragments were cloned on either side of a $Spec^r$ cassette in the *E. coli/S. aureus* shuttle vector pBT2ts. Allelic exchange was performed as previously described (36) and confirmed by sequence analysis. To confirm *spa* deletion, genomic DNA was purified from RN4220 WT, RN4220 Δ*spa::erm*, Newman WT, and Newman Δ*spa::erm*. Primers flanking *spa* were used to amplify across the genomic region around and containing *spa* and result in ≈ 2.1 kb fragments in WT strains and ≈ 1.7 kb fragments in strains where *spa* has been deleted and replaced by *erm*. Primers for amplification across *spa*: Spa.2A: 5′ TTACGCAAGTGTGCTGTATTC Spa.2B: 5′ AACAAAAGATGTTGCTCGTGC. *S. aureus* Δ*srrAB* mutants in RN4220 and Newman were generated as previously described (36).

Real-Time PCR

RNA was purified from log phase (O.D. ≈ 1.0) *S. aureus* by mixing 25 ml of culture with 25 ml of Ethanol/Acetone (1:1). Samples were incubated at −80° C overnight, centrifuged and remaining cell pellets were lysed using Lysing Matrix B (MP Biomedicals, Solon, OH) and bead beater. RNA was purified from lysed cells using the PurLink RNA mini kit (Invitrogen, Carlsbad, CA) following the manufacturers instruction. Quantitative real-time PCR was carried out using the SensiFAST SYBR and Fluorescein One-step kit (Bioline, Taunton,

MA) following manufacturer instructions. The *S. aureus* housekeeping gene rpoD was used to normalize *spa* transcript between strains. Primers: rpoD_RT1.A: 5′ AACTGAATCCAAGTGATCTTAGTG rpoD_RT1.B: 5′ TCATCACCTTGTTCAATACGTTTG spa_RT.1A: 5′ GTAGGTATTGCATCTGTAAC sap_RT.1B: 5′ TTGAGCTTCATCGTGTTGCGC

Kidney burden analysis

To assess the bacterial load in kidneys, 7–8 week-old C57BL/6 mice were infected (iv) with 1×10^6 CFU of *S. aureus* Newman or RN4220 WT, Δ*spa*, or Δ*srrAB*. Kidneys were harvested 5, 7 or 14 days later. Kidneys were homogenized in 1x PBS and serial dilutions of homogenate were plated on BHI. CFUs were counted after overnight incubation at 37°C.

ELISA

For *S. aureus* ELISAs, vinyl plates were coated overnight at 4°C with *S. aureus* RN4220 Δ*spa* grown to stationary phase, washed, and diluted 1:10 in 1x PBS. Plates were blocked with 0.5% bovine serum albumin (BSA) overnight at 4°C. Samples were loaded in 0.5% BSA and incubated overnight at 4°C, then incubated with anti-IgG, anti-IgG2, or anti-IgM conjugated to alkaline phosphatase for 1 hour at 37°C and developed with p-nitrophenyl phosphate. For IsdB ELISAs, polystyrene plates were coated with 3 μg/ml IsdB (AA40-614) in carbonate buffer overnight at 4° and blocked in 0.5% BSA for 1 hour at 37°C. Plates were loaded and developed as for whole *S. aureus* ELISAs. For ClfA ELISAs, polyvinyl plates were coated with 10 μg/ml ClfA in PBS overnight at 4° and blocked in 0.5% BSA for 1 hour at 37°C. Plates were loaded and developed as for whole *S. aureus* ELISAs.

Antibody-mediated macrophage opsophagocytosis assay

GFP-expressing RN4220 (Alexander Horswill, Carver College of Medicine) were incubated with heat-inactivated sera (1% of total reaction) for 10 minutes at room temperature, then incubated with RAW 264.7 macrophages (MFs) at a ratio of 1:15 with rotation at 37° for 20 minutes. After incubation, MFs were treated with trypsin, washed with cold PBS, and fixed in 4% paraformaldehyde. The mean fluorescent intensity of GFP in each sample was determined by flow cytometry.

Flow cytometry

Spleens or lymph nodes were crushed into single cell suspensions in cold 1x PBS. Bone marrow cells were harvested from femurs by flushing with 1x PBS. Cells were washed and red blood cells (RBCs) lysed with 0.165M NH_4Cl. 10^6 cells per sample were labeled with a live/dead dye and Fc receptors blocked with 2.4G2 before Ab staining. Lymphocytes and monocytes were identified by forward and side-scatter profiles. GC B cells were identified as $CD19^+$ $GL7^+$ FAS^+; Tfh cells as $CD4^+$ $PD\text{-}1^+$ $CXCR5^+$; ASCs as $CD138^+$ $B220^{lo/neg}$; DCs as $CD11c^{hi}$ $CD19^-$ $CD3^-$ $CD49b^-$ $Ter119^-$ and $CD8\alpha^+$ or $CD8\alpha^-$; pDCs as $CD11c^+$ $CD19^-$ $CD3^-$ $CD49b^-$ $Ter119^-$ $CD45RA^+$; eosinophils as $Siglec\text{-}F^+$ $CD11b^{int}$. For intracellular cytokine analysis, cells were fixed and permeabilized (BD Biosciences) overnight at 4°C before staining. For intracellular IL-12, cells were incubated for 3 hours at 37°C with 3 ug/ml Brefeldin A before fixing. For chemokine receptor analysis, cells were

labeled in azide-free buffer at 37°C, then washed in normal buffer on ice to halt recycling. Data for DC analyses were collected on a Becton Dickinson LSR II and analyzed with FlowJo. All other samples were run on a Beckman Coulter CyAn ADP and analyzed with Dako Summit.

Antibodies and reagents

Abs specific for CD19, CD3e, CD4, PD-1, APRIL, CD11c, IL-12p40, Ter119, CD11c, MHCII IA/IE, TACI were purchased from Biolegend; GL7, FAS, CXCR5, CD138, B220, IgG, Siglec, CD19, CD8, CD45RA, Annexin V, and Streptavidin-HRP from BD Biosciences; GR-1, CXCR4, CD3e, CD49b, CD40, CD86, CD80 from eBiosciences; BCMA from R&D Systems. IgM (B7.6) and anti-Fc (2.4G2) were purified from hybridomas. Live/Dead Pacific Orange, Fixable Blue dyes, anti-IgG-Alkaline Phosphatase, anti-IgM- Alkaline Phosphatase, Streptavidin were purchased from Invitrogen/Life Technologies; SDF-1 from Shennendoah Biotechnology; anti-IgG2- Alkaline Phosphatase from Southern Biotech; p-Nitrophenyl Phosphate tablets, 3-amino-9-ethylcarbazole tablets, PNA-biotin, and lipopolysaccharide from Sigma; anti-IgGFc from Jackson Immunoresearch; Brefeldin A from LC Laboratories. IsdB peptide (AA 40-613) was a gift from Dr. Benfang Lei (Montana State University). Pokeweed mitogen was a gift from Dr. Shane Crotty (La Jolla Institute for Allergy & Immunology).

ELISpot for ex vivo ASC analysis

Multiscreen ELISpot plates were coated with 1.5 μg/ml IsdB (AA40-613) or NP_{13}BSA in 1x PBS overnight at 4°C and blocked in 1% BSA overnight at 4°C. Single cell suspensions of RBC-lysed splenocytes or BM cells were plated at 0.5–2 × 10^6 cells/well for 18 hours and spots developed with anti-IgG or IgM as previously described (37).

ELISpot for memory B cells

Spleens and lymph nodes were harvested and single cell suspensions were washed and RBC-lysed. Cells were plated at 0.5 × 10^6 cells/ml 1:1 with irradiated (1200 rad) feeder cells and cultured with 5 μg/ml LPS, 1:10,000, and 1:1000 fixed *S. aureus* for 5 days at 37°C. After 5 days, cells were washed and plated in serial densities (0.25–2 × 10^6 cells/well) on IsdB-coated ELISpot plates and developed as above.

Tissue immunofluorescence and microscopy

Spleens were fixed in 4% paraformaldehyde for 5hrs at RT, washed with PBS, hardened in 30% sucrose overnight, and then washed and flash frozen in OCT (Tissue-Tek). Sections (6 microns) were fixed in 1:1 MeOH/acetone and blocked in 0.5% rat serum and 2.4G2 before staining for B220-Alexa647 and IgM-biotin/streptavidin-488 to detect extrafollicular foci. To detect GCs, fixed sections were blocked in 10% FBS and 2.4G2 and stained with B220-Alexa647 and PNA-biotin/streptavidin-488. Sections were imaged with an Olympus Fluoview 500 (10x objective; numerical aperture of 0.45) and analyzed using ImageJ.

Migration assays

Splenic B cells were purified by negative selection (Stem Cell Technologies), and 5×10^6 cells were plated in the top chamber in a 5 μm trans well insert (Corning). For control wells, cells were plated in the bottom chamber with no insert. 100 ng/ml of CXCL12/SDF-1 was added to the bottom chambers and plates were incubated for 3hr at 37°C. Samples from the bottom and control wells were harvested and labelled with CD138 and B220 and enumerated by flow cytometry for 120 seconds per sample. The number of migrated ASCs enumerated from the bottom of the trans well was divided by the number enumerated from the corresponding control well and expressed as a percent of ASCs in the control well.

In vivo proliferation assay

Inoculated and challenged mice were injected (ip) with 2 mg BrdU (Sigma) in PBS on day 10 post-challenge (day 45). After 2 hours, BM cells were harvested and BrdU-labelled ASCs were identified by flow cytometry according the BD Pharmingen BrdU Flow Kit protocol.

Results

S. aureus Protein A interferes with the accumulation of bone marrow plasma cells

To assess whether *S. aureus* induces the formation of LLPCs, and whether this is impacted by Protein A (SpA), we subcutaneously (sc) inoculated mice with strain RN4220 expressing SpA (WT), or an isogenic *spa* deletion mutant (Δ*spa*) (Figure 1A and Supplemental Figure 1A). We chose sc inoculation because clearance of *S. aureus* could be readily monitored at the injection site within 3 weeks. We used the sub-infectious strain, RN4220, because its low virulence allowed mice to be analyzed over a long period of time without significant weight loss or morbidity, while still inducing a localized skin infection and a *S. aureus*-specific Ab response. SpA did not change the frequency of B cells in nearby peripheral lymph nodes (PLNs) after sc infection (Supplemental Figure 1B). Total *S. aureus*-specific serum IgG and IgG2 were detectable between 2 and 5 weeks after primary inoculation with either *S. aureus* Δ*spa* (1.5-fold over naïve) or WT (2-fold over naïve) (Supplemental Figure 1C and D), indicating that our model results in a productive primary B cell response in the presence or absence of SpA.

Most LLPCs are derived from PBs and PCs (hereafter referred to collectively as ASCs) that have taken up residence in the BM within 2 weeks after activation of memory B cells (19, 20). Therefore, we measured the frequencies of ASCs (defined as $CD138^+/B220^{lo/neg}$) in the BM 35 days after inoculation and periodically for 2 weeks following a second intravenous (iv) inoculation (herein referred to as challenge) (Figure 1A). ASCs appeared in the BM by day 40 (day 5 post-challenge), regardless of whether mice were challenged with WT or Δ*spa S. aureus*; however, after on day 45 (day 10 post-challenge) their frequency began to decline in the WT, but not Δ*spa*-challenged mice (Figure 1B). This was not due to an inability to migrate toward the BM niche, since ASCs that formed after WT challenge migrated through a trans-well membrane towards CXCL12 (Supplemental Figure 1E), and expressed similar levels of CXCR4 (Supplemental Figure 1F). The frequencies of total ASCs continued to increase in the BM of Δ*spa*-challenged mice for 2 weeks at which point they were significantly higher than those measured in WT-challenged mice (Figure 1B). This

phenotype was not unique to RN4220, as mice infected and challenged with the clinical strain Newman displayed significantly more BM ASCs 2 weeks after challenge with Δ*spa* Newman compared to WT Newman (Figure 1C). Few ASCs were observed in the BM after primary inoculation (Figure 1B), consistent with studies showing that higher numbers of BMPCs are formed during secondary responses (20, 38). In contrast to the BM phenotype, the frequency of splenic ASCs on day 40 (day 5 post-challenge) was significantly higher in WT- compared to *Δspa*-challenged mice (Figure 1D). No significant differences were observed after day 42 (day 7 post-challenge) when the frequency of ASC began to decline. Therefore, the presence of SpA in the challenge inoculation enhances the splenic ASC response and simultaneously disrupts the accumulation of ASCs in the BM.

IgG Abs specific for the *S. aureus* cell surface hemoglobin receptor IsdB have been detected in infected mice and have recently been studied in vaccine trials (39, 40). To compare the numbers of *S. aureus*-specific ASCs in WT and Δ*spa* challenged mice, we performed ELISpots using IsdB as a target antigen. We observed significantly more IsdB-specific IgG ASCs in the BM of mice challenged with Δ*spa* compared to WT *S. aureus* (Figure 1E), indicating the response is specific to *S. aureus*. In the spleen, IsdB-specific ASCs were found in slightly higher numbers after WT challenge, although this difference was not statistically significant (Figure 1F). Together, these data indicate that primary and early secondary *S. aureus*-specific Ab responses are not quantitatively enhanced in the absence of SpA. These findings are consistent with a previous study (41), but contrary to a study showing that primary iv infection with SpA-deleted *S. aureus* (Newman) enhances the production of Abs to select antigens (42).

S. aureus Protein A does not affect memory B cell responses

The significantly higher numbers of IsdB-specific ASCs observed in the BM 14 days after challenge (day 49) compared to 14 days after primary inoculation is consistent with a memory B cell response (Figure 1E). ASCs that migrate and persist within survival niches in the BM are most often derived from activated memory B cells (19, 43). Therefore, a defect in the early memory responses could have disrupted accumulation of BM ASCs after WT challenge. To test this, we used ELISpot to detect the numbers of IsdB-specific memory B cells after *in vitro* polyclonal stimulation. On day 35 post-inoculation, *ex vivo* lymphocytes from the spleens and lymph nodes were activated with pokeweed mitogen. The 6-day culture period is too short to induce class-switch; thus, all antigen-specific IgG-secreting cells detected by ELISpot are derived from memory B cells (22, 23). We detected significantly greater numbers of memory-derived ASCs in both Δ*spa* and WT-inoculated compared to naïve mice, regardless of whether SpA was present (Figure 2A). Consistent with this, we found that germinal center (GC) B cells, which are required in the formation of high-affinity memory B cells (44), were present after inoculation in the draining lymph nodes and spleen, and appeared with similar kinetics in both WT and Δ*spa* infected mice (Figures 2B–C). T follicular helper cells (Tfh), which are required for GC development (45) were also induced at similar frequencies after primary inoculation with *S. aureus* Δ*spa* or WT (Supplemental Figure 1G).

Memory B cells could be reactivated because secondary IgG2 (IgG2a, 2b, and 2c) responses were more rapid and robust 2 weeks post-challenge compared to a primary responses to the same stimuli (2–6 fold; Figure 2C) (46, 47). This increase was also apparent in total IgG (Supplemental Figure 1H), and dependent on a previous inoculation as indicated by the significantly higher levels of IgG2 and IgG compared to primary inoculation (iv) of naïve mice (Figure 2D and Supplemental Figure 1I). In addition, we found that challenge with either WT or Δ*spa S. aureus* induced early amnestic IgG responses to IsdB (Figure 2E), which were also dependent on previous inoculation (Figure 2F). To ensure that the secondary response was not unique to RN4220, we inoculated and challenged mice with the clinical strains, Newman and USA300. We observed enhanced secondary responses similar to those induced by RN4220 (Figure 2G). Memory to *S. aureus* was long-lived since mice were able to produce amnestic *S. aureus*-specific Ab responses (2–3 fold) when challenged 100 days after primary inoculation (Supplemental Figure 1J).

It was recently shown that when SpA is neutralized during infection, mice produce a more diverse Ab repertoire (48). Thus, despite Δ*spa*-challenged mice making fewer total splenic ASCs than WT-challenged mice, it is possible they developed better Ab diversity, which would enhance *ex vivo* opsonization. We found that heat-inactivated serum from both Δ*spa* and WT challenged mice facilitated phagocytosis of GFP^+ *S. aureus*; however, sera from Δ*spa* challenged mice resulted in enhanced internalization (Figure 2H). Together these data indicate that the lack of accumulation of BM ASCs in WT-challenged mice is not caused by defects in B cell memory formation, or the immediate memory response. Rather, inoculation with *S. aureus* induces memory B cells that respond to challenge by making functional Ab, which is more effective when produced in the absence of SpA.

The presence of Protein A during challenge expands plasmablasts in the spleen

Although mice challenged with WT *S. aureus* (day 35) made significantly more splenic ASCs (day 40) than those challenged with Δ*spa* (Figure 1D), both groups produced similar levels of Abs against *S. aureus* surface proteins in the first 2 weeks after challenge (Figure 2). Both WT and Δ*spa*-challenged mice also displayed similar frequencies of ASCs in the BM for the first 10 days after challenge (Figure 1B). To understand why splenic ASCs induced during WT challenge didn't promote a more robust humoral response, we examined them in greater detail during the peak (day 40) of secondary ASC response (Figure 3A). We found that the expansion of ASCs in WT-challenged mice was not due to changes in the frequency of B cells (Supplemental Figure 2A), but was due to increases in $CD138^+$/$B220^{lo/neg}$ cells (Figure 3B and 3C). Compared to naïve mice given *S. aureus* as a primary inoculation, mice challenged (iv) with either WT or Δ*spa S. aureus* had a higher frequency and total number of ASCs (Figure 3C and Supplemental Figure 2B) and produced 2–6 times more IsdB-specific IgG-secreting ASCs compared to naive mice given a primary inoculation on the same day (Figure 3D). This is consistent with reactivation of memory B cells. In contrast with the frequency of total ASCs, the number of IsdB-specific ASCs was not altered by the presence of SpA, consistent with our findings that short-term secondary IgG responses were similar between WT and Δ*spa* challenged mice (Figure 2). These data suggest that SpA promotes expansion of total ASCs, but not ASCs specific for the *S. aureus* surface antigen, IsdB.

SpA has previously been identified as a B cell superantigen that binds the V_H3 class of BCRs and drives polyclonal B cell expansion and apoptosis (49). Since IgM binds SpA with a higher affinity than IgG (50), we reasoned that if SpA induced the expansion of ASCs by binding Fabs of V_H3 BCRs, the ASC population would contain a large fraction of PBs retaining surface IgM. We found that 54% of the ASCs formed after WT challenge were surface IgM-expressing PBs, compared to only 30% of ASCs formed after Δ*spa* challenge (Figure 3E). The frequency of IgG-expressing PBs was not significantly different between the two groups (20.5% of Δ*spa* and 17.8% of WT) (Figure 3F). These data suggest that most of the surface Ig^+ PBs expanded by SpA during WT challenge were IgM^+ cells that did not result in greater levels of *S. aureus*-specific serum IgM, or increased numbers of IsdB-specific IgM^+ ASCs (Supplemental Figure 2C and 2D). This is consistent with previous observations that SpA^+ *S. aureus* can drive non-specific PB proliferation without inducing Ab secretion (51).

Since the loss of SpA reduces bacterial virulence and colonization of the kidney (52), it is possible that the reduced expansion of IgM^+ PBs during *Δspa* challenge reflects reduced virulence of the *Δspa* mutant. *S. aureus* lacking the virulence factor SrrAB displays a defect in virulence comparable to Δ*spa* (Supplemental Figure 3A). Loss of *srrab* did not disrupt expression of *spa* (Supplemental Figure 3B), allowing us to test the effect of SpA on the expansion of ASC and migration to the BM apart from its effects on bacterial burden. We challenged mice with RN4220 WT, *Δspa*, or Δ*srrAB* and found both WT and Δ*srrAB* expanded ASCs and IgM^+ PBs to levels observed after WT challenge (Supplemental Figure 3C and D). Thus, the lack of expansion of IgM^+ PBs in Δ*spa*-challenge mice was not caused by reduced virulence, but by the absence of SpA.

In models of autoimmunity, sepsis, or chronic inflammation, aberrant expansion of short-lived ASCs has been shown to disrupt the establishment and retention of a LLPC population (53–58). We found that accumulation of BM ASCs was defective in mice challenged with Δ*srrAB* 14 days earlier (Supplemental Figure 3E), and by 14 days post-challenge, no bacteria were found in the kidneys of mice from any group **(data not shown)**. These data demonstrate that virulence or ongoing infection did not cause the observed defect in BM ASC accumulation after WT challenge. Rather, the absence of SpA reduces the expansion of extrafollicular IgM^+ PBs and allows BM ASCs to accumulate.

Protein A induces expansion of short-lived extrafollicular plasmablasts in the spleen

Short-lived ASCs typically divide in the extrafollicular space of lymphoid organs (38). In WT-challenged mice, the transient elevation of IgM^+ ASCs suggests SpA induces short-lived cells that cannot survive long-term in the BM. To assess this, we analyzed spleen sections on day 40 (day 5 post-challenge) and found that compared to Δ*spa*, WT-challenged mice induced larger and more frequent extrafollicular foci composed of $IgM^{+/bright}$ cells (Figure 4A). These foci were located in the splenic red pulp (4A **arrows)** and the bridging channels adjacent to B cell follicles (Figure 4A **asterisks)**. The expansion of extrafollicular foci coincided with a significant increase in the frequency of DCs producing IL-12, a cytokine that promotes ASC differentiation (59, 60) (Supplemental Figure 3F).

It is possible that the expansion of short-lived extrafollicular ASCs occurs at the expense of GC formation (53, 61). This could reduce the pool of ASCs selected for long-term survival in the BM since post-GC PCs likely have a selective advantage once they reach the BM (62). We found that at the peak of the ASC response, the frequency of GC B cells was not reduced in WT-challenged mice compared to *Δspa*-challenged mice (Figure 4B). Instead, mice challenged with WT *S. aureus* produced GC B cells at higher frequencies than those challenged with *Δspa*, and both groups displayed similar GC kinetics as the secondary response resolved (Supplemental Figure 4A). By microscopy, we observed that GC structures of similar sizes were formed after Δ*spa* and WT challenge at similar frequencies (Supplemental Figure 4B and data not shown). Thus, the diminished number of BM ASCs following WT-challenge was not caused by a GC defect.

Protein A diminishes proliferation and CD138 expression of bone marrow ASCs

The inability of ASCs to accumulate in the BM following WT challenge (Figure 1B) could be due to defective migration, the death of ASCs in the BM, or a lack of ASC proliferation in the BM. ASCs arrive in the BM at similar frequencies between days 40 and 45, regardless of whether SpA is expressed during challenge (Figure 1B), and migration toward CXCL12 and expression of CXCR4 were not affected by SpA (Supplemental Figure 1E and F). Thus, the inability of WT-challenged mice to accumulate BM ASCs could be due to an inhospitable BM niche. Systemic inflammation depletes BM niche factors like eosinophils and APRIL that are required for LLPC survival (29, 57, 63), so we assessed whether ASCs with specificity other than *S. aureus* could populate the BM during WT or Δ*spa S. aureus* inoculation. We immunized mice with (4-hydroxy-3-nitrophenyl)-acetyl hapten conjugated to Keyhole limpet hemocyanin (NP-KLH), and 35 days later boosted with NP-KLH alone, or boosted and co-infected (iv) with *S. aureus* RN4220 WT or Δ*spa*. We detected similar numbers of NP-specific IgG ASCs in the BM on day 49 (day 14 post-boost), regardless of whether mice were co-infected with *S. aureus* WT or Δ*spa* (Figure 5A). This suggests that iv inoculation with WT *S. aureus* does not disrupt the competence of the BM to support ASCs. In support of this, we found that the frequencies of eosinophils and APRIL$^+$ BM cells were similar between WT and Δ*spa*-challenged mice (Figure 5B–C). These data suggest that the defect in ASC accumulation after WT *S. aureus* challenge was not caused by a disruption in the BM niche environment, but by an alteration intrinsic to the ASC population.

To determine which cell-intrinsic characteristics were responsible for the lack of accumulation of ASCs in the BM, we measured cell surface expression of the receptors on ASCs that detect BM niche factors (TACI, BCMA, and CD138) (28, 64, 65). Although ASCs from *Δspa* and WT-challenged mice displayed similar levels of TACI and BCMA between days 10 and 12 post-challenge (days 45 and 47) (Supplemental Figures 4C and D), ASCs from *Δspa*-challenged mice expressed significantly higher levels of CD138, a maturation marker and receptor for APRIL (Figure 5D). Although APRIL levels in BM cells were unaffected by SpA (Figure 5C), the diminished expression of CD138 might limit the ability of ASCs (formed after WT challenge) to detect APRIL, a niche factor that provides both proliferation and survival signals in the BM niche.

ASCs that migrate to the BM must proliferate to fill the BM niche and compete for survival factors necessary to become LLPCs (27). To determine whether these requirements were defective in ASCs from WT challenged mice, we measured the frequencies of dividing and apoptotic ASCs in the BM between days 10 and 12 post-challenge. Similar frequencies of ASCs from WT and *Δspa*-challenged mice expressed the apoptotic marker Annexin V (Supplemental Figure 4E), suggesting that cell death is not responsible for the inability of ASCs to become LLPCs in the BM of WT-challenged mice. To assess proliferation of BM ASCs, we intraperitonealy (ip) injected mice with BrdU on day 10 post-challenge (day 45). After two hours, we examined BM ASCs and found that more ASCs from *Δspa*-challenged mice had incorporated BrdU compared to ASCs from WT-challenged mice, indicating that more cells had undergone DNA synthesis (Figure 5E). Thus, after their arrival in the BM, ASCs that formed in the presence of SpA are defective in their ability to proliferate, which may prevent them from occupying the BM niche.

Secretion of antigen-specific IgG by LLPCs is disrupted following challenge with WT *S. aureus*

To test the long-term impact of diminished proliferation after WT challenge, and to confirm that ASCs were not simply delayed in arriving to the BM, we measured IsdB-specific ASCs 8 weeks after challenge (day 91). BM ASCs induced by Δ*spa* challenge were maintained for 8 weeks, indicating they had established a pool of LLPCs; however, the low numbers of ASCs present 2 weeks after WT challenge (day 49) remained unchanged at 8 weeks post-challenge (Figure 6A). Although BM is the main site of LLPC survival, the spleen also harbors PCs for a significant period of time (66). We examined whether expanded ASCs induced by WT challenge could persist in the spleen over time. The numbers of ASCs present in the spleen declined in both WT and Δ*spa*-challenged mice by week 8 post-challenge, to levels that were not different from those in naïve mice (Figure 6B). This indicates that WT challenge did not establish LLPCs in the spleen.

The function of LLPCs is to maintain antigen-specific Abs for long periods of time as an early defense during re-exposure to pathogens (31, 32). We observed that short-term, the *S. aureus*-specific IgG responses in the first 2 weeks after challenge with WT or Δ*spa* were similar (Figure 2). Since we could not detect significant numbers of antigen-specific BM ASCs in WT-challenged mice, we hypothesized that this Ab response would not be maintained beyond two weeks. To test this, we measured serum IgG in groups of WT- or Δ*spa*-challenged mice bi-weekly for 12 weeks after challenge. We found that by 4 weeks post-challenge the levels of IsdB-specific IgG diverged; mice challenged with Δ*spa S. aureus* maintained relatively high serum Ab for the 12-week period, while the serum Ab levels in mice challenged with WT *S. aureus* dropped to levels detected in uninfected mice (Figure 6C). We observed a similar divergence in the Ab response to a different *S. aureus* protein, ClfA, at 12 weeks post-challenge (Figure 6D) as well as in mice infected and challenged with Newman strains (Figure 6E). This drop in antigen-specific Ab is consistent with the decreased frequency of antigen-specific ASCs in the BM (Figure 1B). Importantly, the differences in Ab production we observed between WT and Δ*spa*-challenged mice at 10 and 12 weeks post-challenge were not due to differences present early in the secondary response. Therefore, although the presence of SpA during a challenge inoculation does not disrupt the

formation of memory B cells and secondary ASC responses, it impairs the accumulation of BM ASCs coincident with decreasing proliferation and expression of CD138, and prevented long-term maintenance of antigen-specific serum IgG.

Discussion

S. aureus infections commonly recur without inducing protective immunity. Whether this relates to an inability to form or activate memory B cells, or defects in establishing establish LLPCs in the BM has remained unclear. In this study, we show that SpA caused short-lived extrafollicular PBs to expand and dominate the secondary humoral response within the spleen. In addition, the expression of SpA during challenge infection diminishes the proliferation of ASCs that enter the BM, thereby decreasing the LLPC pool, and consequently, long-term Ab production. These findings identify a previously unappreciated mechanism by which SpA disrupts the formation of LLPCs, a critical event in sustaining Ab titers.

Our findings demonstrate a reciprocal relationship between short-lived ASCs and LLPCs. In cases of infection, chronic antigen exposure promotes splenic short-lived ASC proliferation over BM PC by altering chemokine production and responsiveness (57). Similarly, in a model of systemic lupus erythematosus, chronic availability of autoantigen disrupts the responsiveness of splenic ASCs to CXCL12, coincident with an inability of diseased mice to generate BM PC (66). Our study shows that migration and homing of ASCs to the BM were normal following *S. aureus* infection, suggesting that the mechanism that links splenic ASC expansion to reduced LLPC numbers in *S. aureus* infection is unique.

Expanded numbers of splenic ASCs and reduced numbers of BM PCs are also evident in immunized Fc$\gamma^{-/-}$ mice (60). This phenotype is accompanied by an increase in the numbers of IL-12^{+} DCs. IL-12 has been demonstrated to enhance T-independent B cell responses (59) and because the frequency of IL-12-producing DCs is increased after WT, but not *Δspa* challenge it is possible that SpA-induced IL-12 may enhance T-independent B cells responses during *S. aureus* infection. For example, during challenge infection with WT *S. aureus*, the ability of SpA to bind Fc regions of IgG might impede immune complex-FcγR interactions. The absence of FcγR activation would result in a net increase of DCs producing IL-12 in response to TLR ligands on *S. aureus* (60, 67, 68). In turn, this would induce a preference toward short-lived extrafollicular ASCs over LLPC.

The altered fate of LLPCs after challenge with SpA^{+} *S. aureus* may also be controlled at the level of transcription. Previous work showed that lowering the threshold of B cell activation through loss of the transcription factor Aiolos increases proliferation of short-lived splenic ASCs and diminishes the number of LLPCs in the BM, without affecting GCs, BM niche factors, or chemokine homing (69). This is much like the phenotype we observed in mice challenged with SpA^{+} *S. aureus.* This raises the possibility that SpA ligation of the BCR might alter transcription factors involved in LLPC proliferation or survival.

The ASCs formed after challenge with SpA-deficient *S. aureus* were more likely to proliferate upon reaching the BM (Figure 5E). This phenotype was coincident with an

increase in the levels of CD138, a marker of maturity and a receptor for the ASC survival factor, APRIL (65, 70). Together, these data are consistent with a model of “imprinted” LLPC fate, in which some ASCs differentiating in the spleen receive BCR- or T cell-derived signals that induce a pattern of gene expression required to become a LLPC in the BM (71, 72). In this model, ASCs expanded by SpA in extrafollicular spaces (Figure 4A) would be at a disadvantage because they may be less likely to receive T cell help. Alternatively, they may lack the appropriate BCR signals required to upregulate CD138, or other as-yet-undefined receptors necessary for BM niche survival. This scenario would be similar to infection with *Plasmodium chabaudi* which drives an expanded T-independent ASC response while inhibiting long-term maintenance of the T-dependent Ab response (53). The fact that most of the expanded ASCs induced by SpA were surface IgM^+ may also be important if the genes expressed during formation of LLPC are downstream of BCR signaling through IgG, but not IgM (73). Another possibility is that the expanded short-lived ASCs induced by SpA occupied vital BM niches long enough that properly “imprinted” ASCs missed a window of opportunity to become established in the long-lived pool. There is evidence that such a window is controlled by transient expansion and activation of chemokine-producing BM niche cells (74). This may explain why the numbers of LLPCs in WT *S. aureus*-challenged mice failed to catch-up to those observed in Δ*spa*-challenged mice several weeks after the splenic ASC response resolved (Figure 6A).

Our finding that challenge with SpA^+ *S. aureus* promotes an expansion of IgM^+ PBs has two important implications. First, the expansion is not accompanied by an increase in total serum IgM or in IgM-producing IsdB-specific ASCs, consistent with previous studies showing that SpA promotes non-specific PB proliferation without inducing Ab secretion (51). This ability of SpA to regulate IgM^+ ASCs might have implications during the LLPC response since IgM-FcμR interactions are required for PC trafficking to the BM after T-dependent immunization (75). FcμR negatively regulates T-independent B cell responses in the spleen (75, 76). Therefore, the ability of SpA to increase IgM^+ ASC numbers without an accompanying increase in secreted IgM could indirectly interfere with the establishment of the LLPC compartment. Second, the expansion of IgM^+ PBs after challenge with SpA^+ *S. aureus* suggests a significant contribution of IgM memory B cells in addition to activation of naïve cells. Changes in gene expression allow memory B cells to divide more rapidly compared to naïve B cells (47). Memory B cells also express higher levels of co-stimulatory molecules and TLRs (77). Thus, the effects of SpA may preferentially target the memory population since SpA-BCR binding increases TLR2 sensitivity, resulting in ASC proliferation without Ab production (51). In addition, SpA mediates BCR-mediated uptake of *S. aureus,* leading to the activation of intracellular TLRs (78). Further, IgM memory B cells are more often positioned within the marginal zone compared to IgG memory cells. Their preferred presence in the marginal zone, where exposure to soluble SpA may be enhanced, may also favor short-lived extrafollicular responses (47, 61, 79). Flow-based methods to detect *S. aureus*-specific memory B cells will allow future studies to discern the respective roles of memory B cell subsets in infection (80).

Secondary B cell responses to SpA^+ *S. aureus* are unable to contribute to pathogen clearance (41, 81–83). The underlying mechanisms have remained unclear; however, it was found that abscess formation is decreased when SpA is neutralized (39, 84) or when mice are first

vaccinated with a mutant form of SpA that cannot bind the Fab or Fc portions of Ab (SpA_{kkaa}) (48). These studies implicate SpA in failed protective immunity. Our study provides mechanistic evidence that SpA disrupts sustained production of Ab by preventing the accumulation of LLPCs. Although we could achieve sustained convalescent Ab levels through vaccination with SpA-deficient *S. aureus*, this strategy does not confer protection upon challenge with SpA-expressing *S. aureus* because SpA manipulates memory responses by altering BCR-mediated responses and sequestering IgG that might otherwise participate in preventing or clearing the infection. We confirmed this by challenging previously infected mice, and then re-challenging (3rd infection) with WT Newman *S. aureus* (10^6 CFUs) 14 weeks later. We found that despite the sustained production of Ab after a second infection with SpA-deficient *S. aureus*, the third infection with SpA^+ *S. aureus* resulted in bacterial organ burdens similar to those sustained by mice with no convalescent Ab (mice who received a second infection with WT *S. aureus*) (data not shown). This occurred despite the Ab being functional *in vitro* (Figure 2H). This suggests that the convalescent levels of protective Ab are not adequate to prevent abscess formation in this model. It is important to point out that murine protection studies with *S. aureus* are complicated by the need to use a burden of bacteria that greatly exceeds levels found in human sepsis (typically 10^4–10^5 bacteria/ml blood). Thus, it is possible that convalescent Ab in mice might be sufficient if a more physiologic bacterial burden could be used in assessing protection. Regardless of this limitation in *S. aureus* challenge models, our study shows that SpA impedes the continued production of Ab by preventing the accumulation of LLPCs, in addition to its earlier role in sequestering existing Ab.

Thus, our data show that *S. aureus* infection can generate B cell memory. Despite this, the presence of SpA during a subsequent infection drives an excess of short-lived PBs that do not enhance the *S. aureus*-specific Ab levels, but rather hinder LLPC production. This data may have implications for vaccines as they suggest that infection with SpA-expressing *S. aureus* can manipulate the B cell response and hinder long-lived humoral immunity even if memory has been successfully established. It would be interesting to test the effect of the SpA_{kkaa} vaccine on the accumulation of LLPC and long-term Ab levels. This could provide insight into whether antibodies against SpA are sufficient to attenuate the negative effects of SpA on the LLPC population during challenge infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Benfang Lei (Montana State University) for generously supplying IsdB peptides, Dr. Shane Crotty for kindly providing Pokeweed mitogen and technical support. We also thank Dr. Donna Culton for thoughtful critique and help with study design, and Dr. SunAh Kang and Dr. Karen McKinnon for technical help with experimental design, and Dr. Jessica Cisewski for statistical advice.

Abbreviations used in this article

ASC Ab-secreting cell

BM	bone marrow
Fab	antigen-binding fragment (of Ig)
LLPC	long-lived plasma cell
PB	plasmablast
PC	plasma cell
SpA	*Staphylococcus* protein A

References

1. Klevens RM, Edwards JR, Gaynes RP. The impact of antimicrobial-resistant, health care-associated infections on mortality in the United States. Clin Infect Dis. 2008; 47:927–930. [PubMed: 18752440]
2. Chang FY, Peacock JE, Musher DM, Triplett P, MacDonald BB, Mylotte JM, O'Donnell A, Wagener MM, Yu VL. Staphylococcus aureus bacteremia: recurrence and the impact of antibiotic treatment in a prospective multicenter study. Medicine (Baltimore). 2003; 82:333–9. [PubMed: 14530782]
3. Lucero CA, Hageman J, Zell ER, Bulens S, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Schaffner W, Fridkin SK. Evaluating the potential public health impact of a Staphylococcus aureus vaccine through use of population-based surveillance for invasive methicillin-resistant S. aureus disease in the United States. Vaccine. 2009; 27:5061–5068. [PubMed: 19576943]
4. Zuo Q, Yang L, Feng Q, Lu D, Dong Y, Cai C, Wu Y. Evaluation of the Protective Immunity of a Novel Subunit Fusion Vaccine in a Murine Model of Systemic MRSA Infection. PLoS One. 2013; 8:e81212. [PubMed: 24324681]
5. Scully IL, Liberator PA, Jansen KU, Anderson AS. Covering all the Bases: Preclinical Development of an Effective Staphylococcus aureus Vaccine. Front Immunol. 2014; 5:109. [PubMed: 24715889]
6. Pozzi C, Wilk K, Lee JC, Gening M, Nifantiev N, Pier GB. Opsonic and protective properties of antibodies raised to conjugate vaccines targeting six Staphylococcus aureus antigens. PLoS One. 2012; 7:e46648. [PubMed: 23077517]
7. Maira-Litrán T, Bentancor LV, Bozkurt-Guzel C, O'Malley JM, Cywes-Bentley C, Pier GB. Synthesis and evaluation of a conjugate vaccine composed of Staphylococcus aureus poly-N-acetyl-glucosamine and clumping factor A. PLoS One. 2012; 7:e43813. [PubMed: 22970144]
8. Stranger-Jones YK, Bae T, Schneewind O. Vaccine assembly from surface proteins of Staphylococcus aureus. Proc Natl Acad Sci U S A. 2006; 103:16942–7. [PubMed: 17075065]
9. Adhikari RP, Ajao AO, Aman MJ, Karauzum H, Sarwar J, Lydecker AD, Johnson JK, Nguyen C, Chen WH, Roghmann MC. Lower antibody levels to Staphylococcus aureus exotoxins are associated with sepsis in hospitalized adults with invasive S. aureus infections. J Infect Dis. 2012; 206:915–23. [PubMed: 22807524]
10. Jacobsson G, Colque-Navarro P, Gustafsson E, Andersson R, Möllby R. Antibody responses in patients with invasive Staphylococcus aureus infections. Eur J Clin Microbiol Infect Dis. 2010; 29:715–25. [PubMed: 20383551]
11. Fritz SA, Tiemann KM, Hogan PG, Epplin EK, Rodriguez M, Al-Zubeidi DN, Bubeck Wardenburg J, Hunstad DA. A serologic correlate of protective immunity against community-onset Staphylococcus aureus infection. Clin Infect Dis. 2013; 56:1554–61. [PubMed: 23446627]
12. Becker S, Frankel MB, Schneewind O, Missiakas D. Release of protein A from the cell wall of Staphylococcus aureus. Proc Natl Acad Sci U S A. 2014; 111:1574–9. [PubMed: 24434550]
13. Bergtold A, Desai DD, Gavhane A, Clynes R. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. Immunity. 2005; 23:503–14. [PubMed: 16286018]

14. Palmqvist N, Silverman GJ, Josefsson E, Tarkowski A. Bacterial cell wall-expressed protein A triggers supraclonal B-cell responses upon in vivo infection with Staphylococcus aureus. Microbes Infect. 2005; 7:1501–11. [PubMed: 16039897]
15. Pauli NT, Kim HK, Falugi F, Huang M, Dulac J, Henry Dunand C, Zheng N-Y, Kaur K, Andrews SF, Huang Y, DeDent AC, Frank KM, Charnot-Katsikas A, Schneewind O, Wilson PC. Staphylococcus aureus infection induces protein A-mediated immune evasion in humans. J Exp Med. 2014; 211:2331–2339. [PubMed: 25348152]
16. Silverman G, Sasano M, Wormsley S. Age-Associated Changes in Binding of Human B Lymphocytes to a VH3-Restricted Unconventional Bacterial Antigen. J Immunol. 1993; 151:5840–5855. [PubMed: 7693819]
17. Forsgren A, Nordstrom K. Protein A from Staphylococcus aureus: The biological significance of its reaction with IgG. Ann NY Acad Sci. 1974:252–266.
18. Peterson PK, Verhoef JAN, Sabath LD, Quie PG. Effect of Protein A on Staphylococcal opsonization. Infect Immun. 1977; 15:760–764. [PubMed: 870431]
19. Hauser AE, Debes GF, Arce S, Cassese G, Hamann A, Radbruch A, Manz Ra. Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. J Immunol. 2002; 169:1277–82. [PubMed: 12133949]
20. Manz RA, Radruch A. Lifetime of plasma cells in the bone marrow. Nature. 1997; 388:133–134. [PubMed: 9217150]
21. Benner R, Hijmans W, Haaijman JJ. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. Clin Exp Immunol. 1981; 46:1–8. [PubMed: 7039877]
22. Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. SAP is required for generating long-term humoral immunity. Nature. 2003; 421:282–286. [PubMed: 12529646]
23. Ndungu FM, Cadman ET, Coulcher J, Nduati E, Couper E, Macdonald DW, Ng D, Langhorne J. Functional memory B cells and long-lived plasma cells are generated after a single Plasmodium chabaudi infection in mice. PLoS Pathog. 2009; 5:e1000690. [PubMed: 20011127]
24. Yates JL, Racine R, McBride KM, Winslow GM. T cell-dependent IgM memory B cells generated during bacterial infection are required for IgG responses to antigen challenge. J Immunol. 2013; 191:1240–9. [PubMed: 23804710]
25. Alam MM, Aktar A, Afrin S, Rahman MA, Aktar S, Uddin T, Rahman MA, Al Mahbuba D, Chowdhury F, Khan AI, Bhuiyan TR, Begum YA, Ryan ET, Calderwood SB, Svennerholm A-M, Qadri F. Antigen-Specific Memory B-cell Responses to Enterotoxigenic Escherichia coli Infection in Bangladeshi Adults. PLoS Negl Trop Dis. 2014; 8:e2822. [PubMed: 24762744]
26. O'Connor BP, Gleeson MW, Noelle RJ, Erickson LD. The rise and fall of long-lived humoral immunity: terminal differentiation of plasma cells in health and disease. Immunol Rev. 2003; 194:61–76. [PubMed: 12846808]
27. O'Connor BP, Cascalho M, Noelle RJ. Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population. J Exp Med. 2002; 195:737–45. [PubMed: 11901199]
28. Tsuji S, Cortesão C, Bram RJ, Platt JL, Cascalho M, O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, Noelle RJ. TACI deficiency impairs sustained Blimp-1 expression in B cells decreasing long-lived plasma cells in the bone marrow. Blood. 2011; 118:5832–9. [PubMed: 21984806]
29. Belnoue E, Pihlgren M, McGaha TL, Tougne C, Rochat AF, Bossen C, Schneider P, Huard B, Lambert PH, Siegrist CA. APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells. Blood. 2008; 111:2755–64. [PubMed: 18180376]
30. Ozcan E, Garibyan L, Lee JJY, Bram RJ, Lam KP, Geha RS. Transmembrane activator, calcium modulator, and cyclophilin ligand interactor drives plasma cell differentiation in LPS-activated B cells. J Allergy Clin Immunol. 2009; 123:1277–86.e5. [PubMed: 19427685]
31. Amanna IJ, Slifka MK. Contributions of humoral and cellular immunity to vaccine-induced protection in humans. Virology. 2011; 411:206–15. [PubMed: 21216425]

32. Taillardet M, Haffar G, Mondière P, Asensio MJ, Gheit H, Burdin N, Defrance T, Genestier L. The thymus-independent immunity conferred by a pneumococcal polysaccharide is mediated by long-lived plasma cells. Blood. 2009; 114:4432–40. [PubMed: 19767510]
33. Bortnick A, Allman D. What is and what should always have been: long-lived plasma cells induced by T cell-independent antigens. J Immunol. 2013; 190:5913–8. [PubMed: 23749966]
34. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral Immunity Due to Long-Lived Plasma Cells. Immunity. 1998; 8:363–372. [PubMed: 9529153]
35. Halliley JL, Tipton CM, Liesveld J, Rosenberg AF, Darce J, Gregoretti IV, Popova L, Kaminiski D, Fucile CF, Albizua I, Kyu S, Chiang K-Y, Bradley KT, Burack R, Slifka M, Hammarlund E, Wu H, Zhao L, Walsh EE, Falsey AR, Randall TD, Cheung WC, Sanz I, Lee FE-H. Long-Lived Plasma Cells Are Contained within the CD19−CD38hiCD138+ Subset in Human Bone Marrow. Immunity. 2015:1–14.
36. Richardson AR, Dunman PM, Fang FC. The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. Mol Microbiol. 2006; 61:927–39. [PubMed: 16859493]
37. Lee SR, Rutan JA, Monteith AJ, Jones SZ, Kang SA, Krum KN, Kilmon MA, Roques JR, Wagner NJ, Clarke SH, Vilen BJ. Receptor cross-talk spatially restricts p-ERK during TLR4 stimulation of autoreactive B cells. J Immunol. 2012; 189:3859–68. [PubMed: 22984080]
38. Smith KGC, Hewitson TD, Nossal GJ, Tarlinton DM, Walter T, Hall E. The phenotype and fate of the antibody-forming cells of the splenic foci. Eur J Immunol. 1996; 26:444–448. [PubMed: 8617316]
39. Falugi F, Kim HK, Missiakas DM. Role of Protein A in the Evasion of Host Adaptive Immune Responses by Staphylococcus aureus. MBio. 2013:4.
40. Fowler VG, Allen KB, Moreira ED, Moustafa M, Isgro F, Boucher HW, Corey GR, Carmeli Y, Betts R, Hartzel JS, Chan ISF, McNeely TB, Kartsonis Na, Guris D, Onorato MT, Smugar SS, DiNubile MJ, Sobanjo-ter Meulen A, Fowler VGBH, Allen KB, Moreira ED, Moustafa M, Isgro F, Corey GRMT, Carmeli Y, Betts R, Hartzel JS, Chan IS, Kartsonis NADM, Guris D, Onorato MT, Smugar SS, SMA. Effect of an investigational vaccine for preventing Staphylococcus aureus infections after cardiothoracic surgery: a randomized trial. JAMA. 2013; 309:1368–1378. [PubMed: 23549582]
41. van Diemen PM, Yamaguchi Y, Paterson GK, Rollier CS, Hill AVS, Wyllie DH. Irradiated wild-type and Spa mutant Staphylococcus aureus induce anti-S. aureus immune responses in mice which do not protect against subsequent intravenous challenge. Pathog Dis. 2013; 68:20–6. [PubMed: 23620394]
42. Kim HK, Kim HY, Schneewind O, Missiakas D. Identifying protective antigens of Staphylococcus aureus, a pathogen that suppresses host immune responses. FASEB J. 2011; 25:3605–12. [PubMed: 21753082]
43. Manz RA, Löhning M, Cassese G, Thiel A, Radbruch A. Survival of long-lived plasma cells is independent of antigen. Int Immunol. 1998; 10:1703–11. [PubMed: 9846699]
44. Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. J Exp Med. 2005; 201:545–554. [PubMed: 15710653]
45. Vinuesa CG, Cyster JG. How T cells earn the follicular rite of passage. Immunity. 2011; 35:671–680. [PubMed: 22118524]
46. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. Science (80-). 1996; 272:54–60.
47. Tangye SG, Tarlinton DM. Memory B cells: effectors of long-lived immune responses. Eur J Immunol. 2009; 39:2065–75. [PubMed: 19637202]
48. Kim HY, Cheng AG, Missiakas DM, Schneewind O. Nontoxigenic protein A vaccine for methicillin-resistant Staphylococcus aureus infections in mice. J Exp Med. 2010; 207:1863–70. [PubMed: 20713595]
49. Silverman GJ, Cary SP, Dwyer DC, Luo L, Wagenknecht R, Curtiss VE. A B cell superantigen-induced persistent “Hole” in the B-1 repertoire. J Exp Med. 2000; 192:87–98. [PubMed: 10880529]

50. Roben PW, Salem AN, Silverman CJ. VH3 Family antibodies bind domain D of Staphylococcal Protein A. J Immunol. 1995; 154:6437–6445. [PubMed: 7759880]
51. Bekeredjian-ding I, Inamura S, Giese T, Moll H, Endres S, Sing A, Za U. B Cell Proliferation by Sensitizing B Cells for TLR2 Ligands. J Immunol. 2007; 178:2803–2812. [PubMed: 17312124]
52. Palmqvist N, Foster T, Tarkowski A, Josefsson E. Protein A is a virulence factor in Staphylococcus aureus arthritis and septic death. Microb Pathog. 2002; 33:239–249. [PubMed: 12473438]
53. Wilmore JR, Maue AC, Lefebvre JS, Haynes L, Rochford R. Acute Plasmodium chabaudi infection dampens humoral responses to a secondary T-dependent antigen but enhances responses to a secondary T-independent antigen. J Immunol. 2013; 191:4731–9. [PubMed: 24081991]
54. Huang H, Benoist C, Mathis D. Rituximab specifically depletes short-lived autoreactive plasma cells in a mouse model of inflammatory arthritis. Proc Natl Acad Sci U S A. 2010; 107:4658–63. [PubMed: 20176942]
55. Tiburzy B, Szyska M, Iwata H, Chrobok N, Kulkarni U, Hirose M, Ludwig RJ, Kalies K, Westermann J, Wong D, Manz RA. Persistent autoantibody-production by intermediates between short-and long-lived plasma cells in inflamed lymph nodes of experimental epidermolysis bullosa acquisita. PLoS One. 2013; 8:e83631. [PubMed: 24386241]
56. Mathian A, Gallegos M, Pascual V, Banchereau J, Koutouzov S. Interferon-α induces unabated production of short-lived plasma cells in pre-autoimmune lupus-prone (NZB×NZW)F1 mice but not in BALB/c mice. Eur J Immunol. 2011; 41:863–72. [PubMed: 21312191]
57. Slocombe T, Brown S, Miles K, Gray M, Barr Ta, Gray D. Plasma cell homeostasis: the effects of chronic antigen stimulation and inflammation. J Immunol. 2013; 191:3128–38. [PubMed: 23935195]
58. Pötschke C, Kessler W, Maier S, Heidecke CD, Bröker BM. Experimental sepsis impairs humoral memory in mice. PLoS One. 2013; 8:e81752. [PubMed: 24312349]
59. Buchanan RM, Arulanandam BP, Metzger DW. IL-12 enhances antibody responses to T-independent polysaccharide vaccines in the absence of T and NK cells. J Immunol. 1998; 161:5525–33. [PubMed: 9820529]
60. Kim SJ, Caton M, Wang C, Khalil M, Zhou ZJJ, Hardin J, Diamond B. Increased IL-12 inhibits B cells' differentiation to germinal center cells and promotes differentiation to short-lived plasmablasts. J Exp Med. 2008; 205:2437–48. [PubMed: 18809711]
61. Pulendran B, Smith KG, Nossal GJ. Soluble antigen can impede affinity maturation and the germinal center reaction but enhance extrafollicular immunoglobulin production. J Immunol. 1995; 155:1141–50. [PubMed: 7543516]
62. Takahashi Y, Dutta PR, Cerasoli DM, Kelsoe G. In Situ Studies of the Primary Immune Response to (4-Hydroxy-3-Nitrophenyl)Acetyl. V. Affinity Maturation Develops in Two Stages of Clonal Selection. J Exp Med. 1998; 187:885–895. [PubMed: 9500791]
63. Chu VT, Fröhlich A, Steinhauser G, Scheel T, Roch T, Fillatreau S, Lee JJ, Löhning M, Berek C. Eosinophils are required for the maintenance of plasma cells in the bone marrow. Nat Immunol. 2011; 12:151–9. [PubMed: 21217761]
64. O'Connor BP V, Raman S, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, Noelle RJ. BCMA is essential for the survival of long-lived bone marrow plasma cells. J Exp Med. 2004; 199:91–98. [PubMed: 14707116]
65. Ingold K, Zumsteg A, Tardivel A, Huard B, Steiner QG, Cachero TG, Qiang F, Gorelik L, Kalled SL, Acha-Orbea H, Rennert PD, Tschopp J, Schneider P. Identification of proteoglycans as the APRIL-specific binding partners. J Exp Med. 2005; 201:1375–83. [PubMed: 15851487]
66. Erickson LD, Lin LL, Duan B, Morel L, Noelle RJ. A genetic lesion that arrests plasma cell homing to the bone marrow. Proc Natl Acad Sci U S A. 2003; 100:12905–10. [PubMed: 14555759]
67. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. Cell. 2006; 124:849–63. [PubMed: 16497593]
68. Schmaler M, Jann NJ, Ferracin F, Landmann R. T and B cells are not required for clearing Staphylococcus aureus in systemic infection despite a strong TLR2-MyD88-dependent T cell activation. J Immunol. 2011; 186:443–52. [PubMed: 21131426]

69. Cortés M, Georgopoulos K. Aiolos is required for the generation of high affinity bone marrow plasma cells responsible for long-term immunity. J Exp Med. 2004; 199:209–19. [PubMed: 14718515]
70. Szyszko, Ea, Skarstein, K., Jonsson, R., Brokstad, KA. Distinct phenotypes of plasma cells in spleen and bone marrow of autoimmune NOD.B10.H2b mice. Autoimmunity. 2011; 44:415–26. [PubMed: 21332424]
71. Amanna IJ, Slifka MK. Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. Immunol Rev. 2010; 236:125–38. [PubMed: 20636813]
72. Wang Y, Bhattacharya D. Adjuvant-specific regulation of long-term antibody responses by ZBTB20. J Exp Med. 2014; 211:841–856. [PubMed: 24711582]
73. Wakabayashi C, Adachi T, Wienands J, Tsubata T. A distinct signaling pathway used by the IgG-containing B cell antigen receptor. Science (80-). 2002; 298:2392–2395.
74. Chu VT, Berek C. The establishment of the plasma cell survival niche in the bone marrow. Immunol Rev. 2013; 251:177–88. [PubMed: 23278749]
75. Choi SC, Wang H, Tian L, Murakami Y, Shin DM, Borrego F, Morse HC, Coligan JE. Mouse IgM Fc receptor, FCMR, promotes B cell development and modulates antigen-driven immune responses. J Immunol. 2013; 190:987–96. [PubMed: 23267023]
76. Honjo K, Kubagawa Y, Jones DM, Dizon B, Zhu Z, Ohno H, Izui S, Kearney JF, Kubagawa H. Altered Ig levels and antibody responses in mice deficient for the Fc receptor for IgM (FcμR). Proc Natl Acad Sci U S A. 2012; 109:15882–7. [PubMed: 22984178]
77. Good KL, Avery DT, Tangye SG. Resting Human Memory B Cells are intrinsically programmed for Enhanced Survival and Responsiveness to Diverse Stimuli Compared to Naive B Cells. J Immunol. 2009; 182:890–901. [PubMed: 19124732]
78. Parcina M, Miranda-Garcia MA, Durlanik S, Ziegler S, Over B, Georg P, Foermer S, Ammann S, Hilmi D, Weber KJ, Schiller M, Heeg K, Schneider-Brachert W, Götz F, Bekeredjian-Ding I. Pathogen-triggered activation of plasmacytoid dendritic cells induces IL-10-producing B cells in response to Staphylococcus aureus. J Immunol. 2013; 190:1591–602. [PubMed: 23325892]
79. Aiba Y, Kometani K, Hamadate M, Moriyama S, Sakaue-sawano A, Tomura M, Luche H, Fehling HJ, Casellas R, Kanagawa O, Miyawaki A, Kurosaki T. Preferential localization of IgG memory B cells adjacent to contracted germinal centers. Proc Natl Acad Sci U S A. 2010; 107:12192–12197. [PubMed: 20547847]
80. Zuccarino-Catania GV, Sadanand S, Weisel FJ, Tomayko MM, Meng H, Kleinstein SH, Good-Jacobson KL, Shlomchik MJ. CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. Nat Immunol. 2014
81. Spellberg B, Ibrahim AS, Yeaman MR, Lin L, Fu Y, Avanesian V, Bayer AS, Filler SG, Lipke P, Otoo H, Edwards JE. The antifungal vaccine derived from the recombinant N terminus of Als3p protects mice against the bacterium Staphylococcus aureus. Infect Immun. 2008; 76:4574–80. [PubMed: 18644876]
82. Joshi A, Pancari G, Cope L, Bowman EP, Cua D, Proctor RA, Mcneely T. Immunization with Staphylococcus aureus iron regulated surface determinant B (IsdB) confers protection via Th17/IL17 pathway in a murine sepsis model. Hum Vaccines Immunother. 2012; 8:336–346.
83. Bröker BM, Holtfreter S, Bekeredjian-ding I. Immune control of Staphylococcus aureus – Regulation and counter-regulation of the adaptive immune response. Int J Med Microbiol. 2014; 304:204–214. [PubMed: 24462009]
84. Kim HK, Emolo C, DeDent AC, Falugi F, Missiakas DM, Schneewind O. Protein A-specific monoclonal antibodies and prevention of Staphylococcus aureus disease in mice. Infect Immun. 2012; 80:3460–70. [PubMed: 22825452]

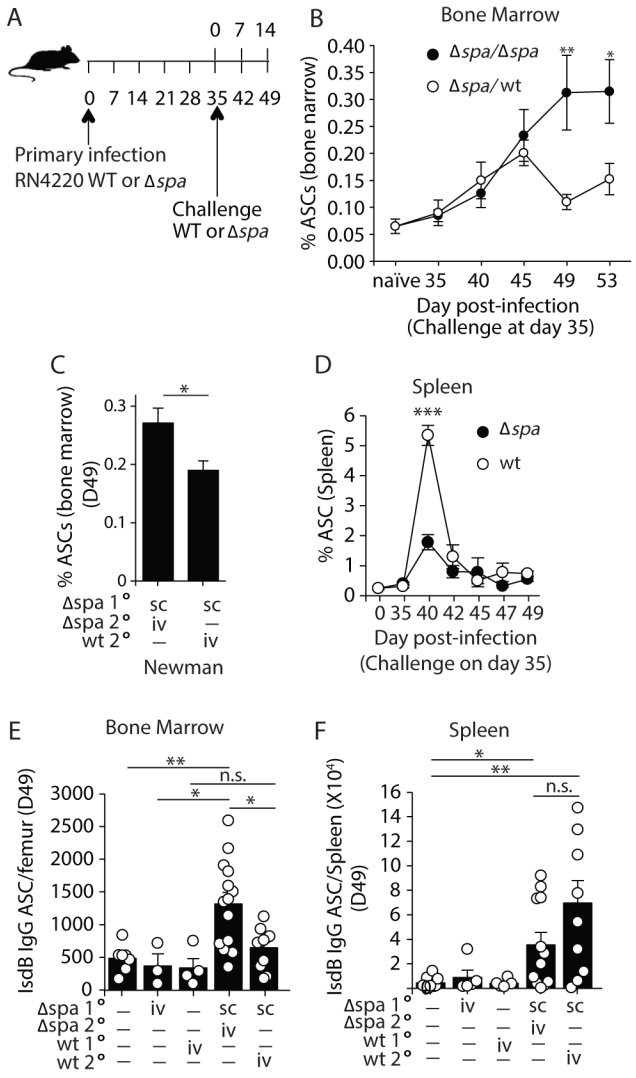

Figure 1. ASCs fail to populate the bone marrow after challenge with Protein A (SpA^{+}) WT *S. aureus*

(A–B, D–F) Mice were inoculated (sc) with *S. aureus* RN4220 WT or Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. **(A)** Schematic of inoculation and challenge course. **(B)** CD138^{+} B220$^{lo/neg}$ ASCs from the BM were quantitated by flow cytometry on the indicated days. n=5 experiments, 3–12 mice/time point. **(C)** Mice were inoculated (sc) with *S. aureus* Newman Δ*spa* and challenged 35 days later with *S. aureus* Newman Δ*spa* or WT. Frequency of BM ASCs was measured on day 49 (day 14 post-challenge). n=2 experiments, 5–7 mice/group. **(D)** The frequency of splenic ASCs at the indicated time points. n=7 experiments, 3–13 mice/time point. **(E–F)** Anti-IsdB IgG-producing ASCs quantitated by ELISpot from (E) BM and (F) spleens on day 49 (day 14 post-challenge). Data represent (E) 7 experiments, 3–14 mice per group and (F) 10 experiments, 5–12 mice per group. Differences in B–D were determined by a Mann Whitney test; for B time points were compared independently. E and F were compared using one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001 n.s.=not significant. Error bars=standard error of the mean.

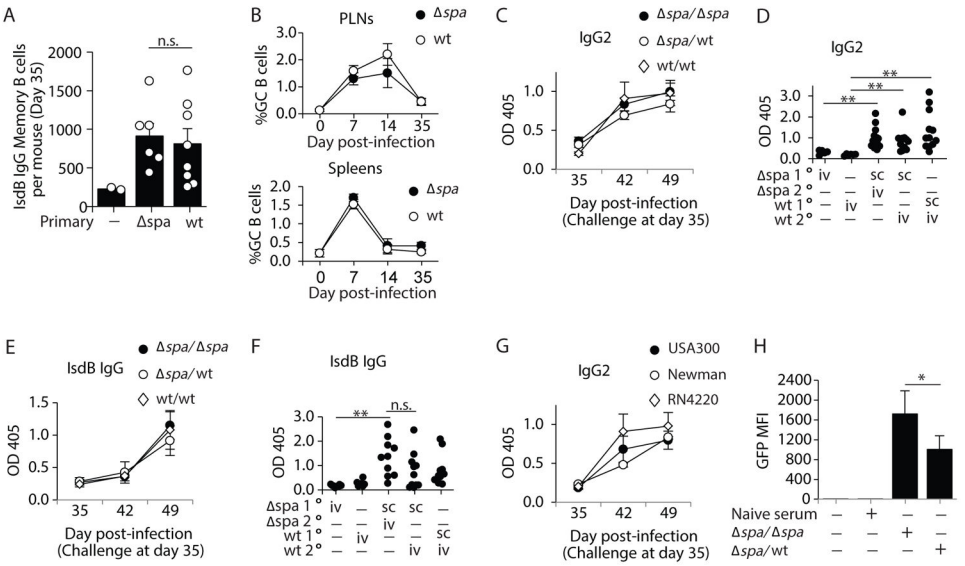

Figure 2. Memory B cells are formed and activated in response to *S. aureus*

(A–H) Mice were inoculated (sc) with *S. aureus* RN4220 WT or Δ*spa*. **(A)** Memory B cells detected by ELISpot 35 days post-inoculation. Data represent 3 experiments, 6–8 mice per group. **(B)** Frequencies of GC B cells in draining PLNs or spleens at indicated time points. Data represent 4 experiments, 3–6 mice per group per time point. **(C–F)** Primed or naïve mice were challenged (iv) with *S. aureus* RN4220 WT or Δ*spa* on day 35. *S. aureus*-specific (C and D) IgG2 or (E and F) IsdB-specific IgG measured by ELISA from sera collected on the indicated days. Sera for D and F were collected on day 14 post-challenge (day 49 for primed mice). Data represent (C) 7 experiments, 9–21 mice per group, (D) 3 experiments, 5–18 mice per group, (E) 10 experiments with 5–16 mice per group, (F) 3 experiments, 9–12 mice per group. **(G)** *S. aureus*-specific serum IgG from mice inoculated (sc) with *S. aureus* RN4220, Newman, or USA300 and challenged with the same strain 35 days later. Data represent 3 experiments, 3–8 mice per group. **(H)** Mean fluorescence intensity (MFI) of GFP-expressing *S. aureus* after *in vitro* phagocytosis by macrophages in the presence or absence of day 49 serum harvested from mice infected and challenged as in A. Data represent 4 experiments, 2–8 mice per group. O.D.=optical density. Groups in A, C, and E were compared using a Mann Whitney test; D and F using one-way ANOVA; H using an unpaired t test. *p<0.05, **p<0.01, n.s.=not significant. Error bars=standard error of the mean.

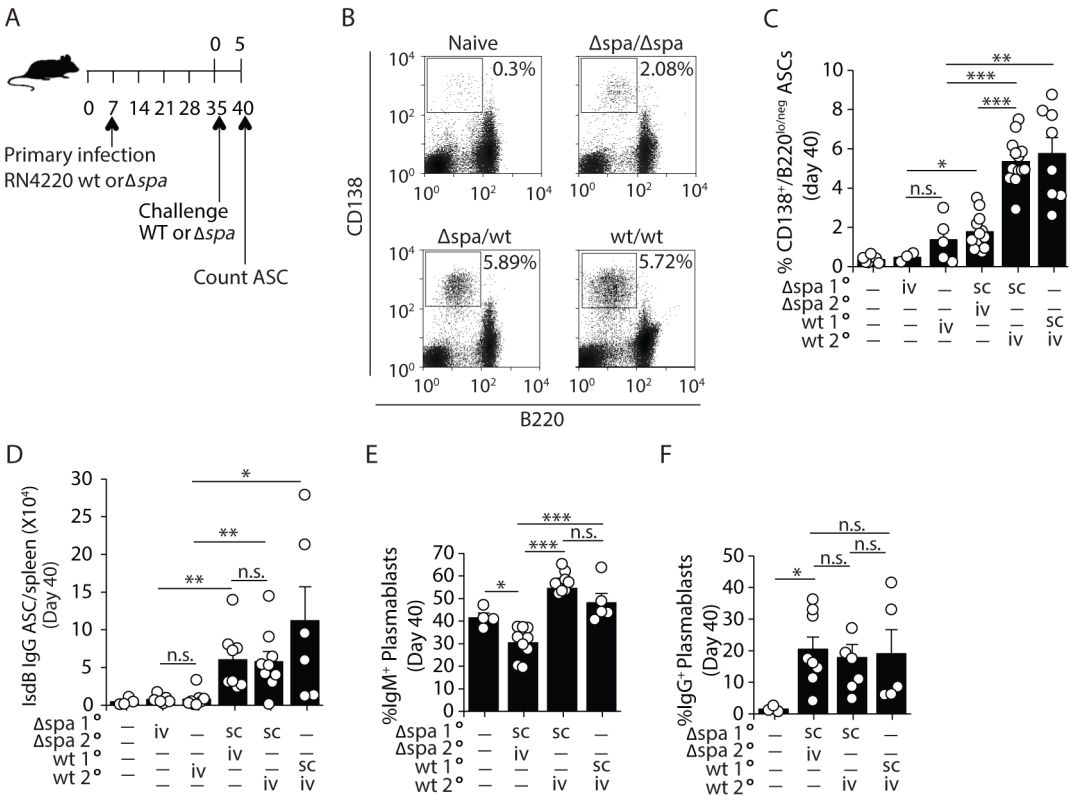

Figure 3. SpA induces the expansion of IgM$^+$ plasmablasts

(A) Schematic of inoculation and challenge course. (A–F) Mice were inoculated (sc) with *S. aureus* RN4220 WT or Δ*spa*. On day 35, primed and naïve mice were challenged (iv) with *S. aureus* RN4220 WT or Δ*spa*. Spleens were harvested for analysis 5 days later (day 40). **(B)** Representative flow cytometry histograms of ASC analysis in (C). **(C)** Compilation of the frequency of ASCs from (B). Data represent 7 experiments, 2–13 mice per group. **(D)** Anti-IsdB IgG-producing ASCs quantitated by ELISpot. n=4 experiments, 4–9 mice/group. **(E–F)** Frequency of surface (E) IgM$^+$ and (F) IgG$^+$ ASCs measured by flow cytometry. n=4 experiments, 3–9 mice/group. Groups in C–F using one-way ANOVA. FS=forward scatter; SS=side scatter; *p<0.05, **p<0.01, ***p<0.001, n.s.=not significant. Error bars=standard error of the mean.

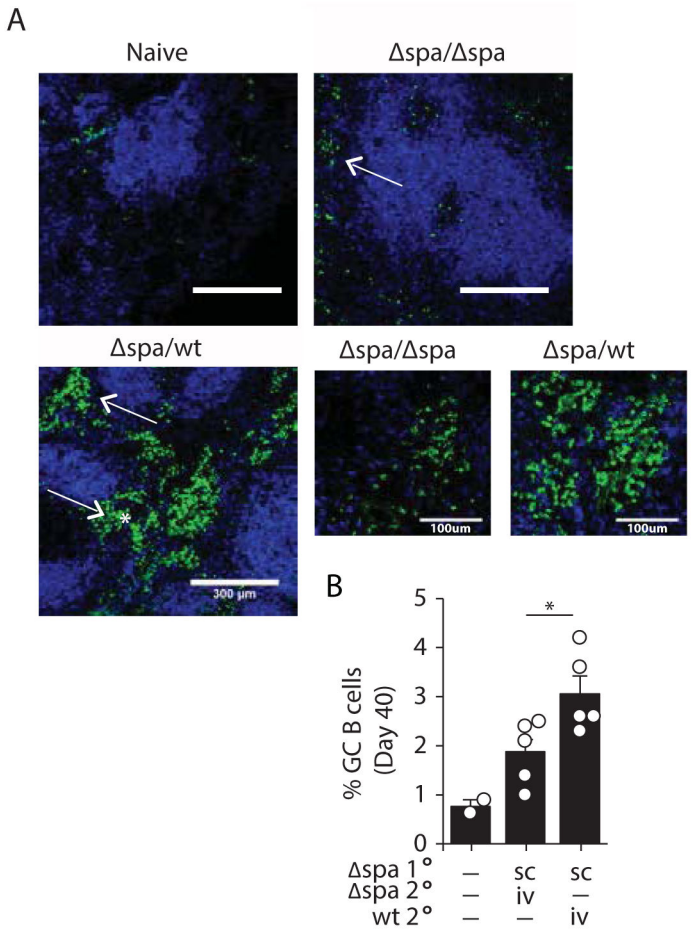

Figure 4. SpA drives an expanded extrafollicular response

(A–B) Mice were inoculated (sc) with *S. aureus* RN4220 Δ*spa,* and challenged (iv) 35 days later with *S. aureus* RN4220 Δ*spa* or WT. **(A)** Day 40 spleen sections labeled with B220 (blue) and IgM (green) to image extrafollicular foci. n=3 experiments, 5–6 mice/group. Arrows indicate foci and * indicates bridging channels. Full image scale bar=300 microns, inset scale bars=100 microns. **(B)** The frequencies of splenic GC B cells measured on day 40 (day 5 post-challenge). n=2 experiments, 2–5 mice/group. Groups in B were compared using a Mann Whitney test. n.s.=not significant. Error bars=standard error of the mean.

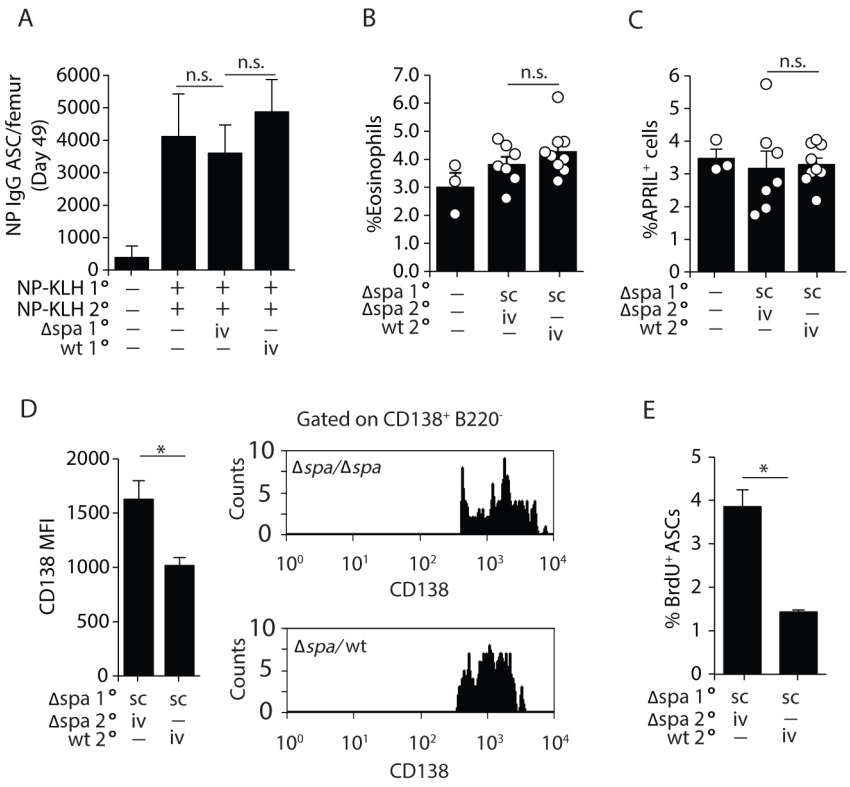

Figure 5. The presence of SpA during challenge induces defects in the proliferation of bone marrow ASCs and the expression of CD138

(A) Mice were immunized with NP_{20}KLH by i.p. injection and 35 days later boosted with soluble NP_{20}KLH alone or co-infected (iv) with *S. aureus* RN4220 Δ*spa* or WT. On day 49 (day 14 post-boost/inoculation), NP-specific IgG-producing ASC were quantitated by ELISpot. n=2 experiments, 2–6 mice/group. **(B–E)** Mice were inoculated (sc) with *S. aureus* RN4220 Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. **(B–C)** The frequency of (B) eosinophils and (C) APRIL-producing cells present in BM on day 40 (day 5 post-challenge). n=3 experiments, 3–6 mice/group. **(D)** MFI and representative flow cytometry histograms of CD138 on BM ASCs on days 45 and 47 (days 10 and 12 post-challenge). n=3 experiments, 6–10 mice per group. **(E)** Frequency of $BrdU^+$ BM ASCs on day 10 post-challenge (day 45). n=2 experiments, 6 mice/group. Groups in A were compared using one-way ANOVA; B–C using a Mann Whitney test; for D and E, an unpaired t test was used. *p<0.05, n.s.=not significant. Error bars=standard error of the mean.

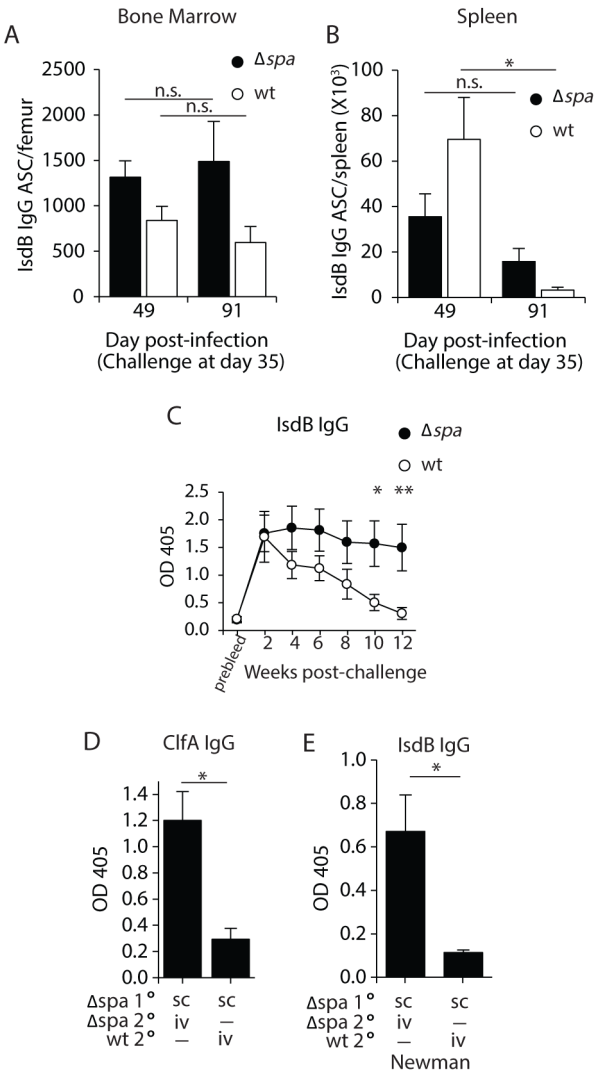

Figure 6. SpA prevents long-term maintenance of bone marrow LLPCs and serum Ab

(A–D) Mice were inoculated (sc) with *S. aureus* RN4220 Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. **(A–B)** Anti-IsdB IgG-producing ASCs quantitated by ELISpot from the (A) BM and (B) spleens of challenged mice on days 49 and 91 (day 14 and 56 post-challenge). n=3 experiments, 3–6 mice/group. **(C)** IsdB-specific IgG measured by ELISA from sera collected on indicated time points after challenge. n=5 experiments, 4–10 mice/group. **(D)** ClfA-specific IgG measured by ELISA from sera collected 12 weeks post-challenge (day 119). n=4 experiments, 5–6 mice per group. **(E)** Mice were inoculated (sc) with *S. aureus* Newman Δ*spa* then challenged 35 days later with *S. aureus* Newman WT or Δ*spa.* IsdB-specific IgG was measured by ELISA from sera collected 14 weeks post-challenge (day 133). n=3 experiments, 4–8 mice/group. Differences in A–E were determined by a Mann Whitney test; for C time points were compared independently. OD=optical density, *p<0.05, n.s.=not significant. Error bars=standard error of the mean.